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RESEARCH PAPER

Enzyme activities and subcellular localization of members of the *Arabidopsis* glutathione transferase superfamily

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Abstract

Enzyme screens with *Strep*-tagged recombinant proteins and expression studies with the respective green fluorescent protein (GFP) fusions have been employed to examine the functional activities and subcellular localization of members of the *Arabidopsis* glutathione transferase (GST) superfamily. Fifty-one of 54 GST family members were transcribed and 41 found to express as functional glutathione-dependent enzymes in *Escherichia coli*. Functional redundancy was observed and in particular three theta (T) class GSTs showed conserved activities as hydroperoxide-reducing glutathione peroxidases (GPOXs). When expressed in tobacco as GFP fusions, all three GSTTs localized to the peroxisome, where their GPOX activity could prevent membrane damage arising from fatty acid oxidation. Through alternative splicing, two of these GSTTs form fusions with Myb transcription factor-like domains. Examination of one of these variants showed discrete localization within the nucleus, possibly serving a role in reducing nucleic acid hydroperoxides or in signalling. Based on this unexpected differential sub-cellular localization, 15 other GST family members were expressed as GFP fusions in tobacco. Most accumulated in the cytosol, but GSTU12 localized to the nucleus, a family member resembling a bacterial tetrachlorohydroquinone dehalogenase selectively associated with the plasma membrane, and a lambda GSTL2 was partially directed to the peroxisome after removal of a putative chloroplast transit peptide. Based on the results obtained with the GSTTs, it was concluded that these proteins can exert identical protective functions in differing subcellular compartments.

Key words: Alternative splicing, confocal microscopy, glutathione peroxidase, green fluorescent protein, lipid hydroperoxides, *Nicotiana benthamiana*, peroxisome, *Strep* tag.

Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) in plants are a superfamily of proteins which can be clustered into six groupings based on similarities in sequence and gene organization, namely the phi (F), tau (U), theta (T), zeta (Z), lambda (L), and dehydroascorbate reductase (DHAR) classes (Dixon *et al.*, 2002b). Recent informatic studies in *Arabidopsis thaliana* (L.) Heynh have also revealed a further single representative of an additional type of GST which most closely resembles the bacterial tetrachlorohydroquinone dehalogenase (TCHQD) (Edwards and Dixon, 2005). Recently progress has been made in defining the function of some of the smaller groups of plant GSTs. Thus GSTZs appear to function as glutathione (GSH)-dependent isomerases in tyrosine catabolism (Dixon and

Edwards, 2006), while the DHARs are active in ascorbic acid recycling (Dixon *et al.*, 2002a). By contrast, the functions of the majority of plant GSTs remain unknown. Using *Arabidopsis* as an example, individual GSTs have been shown to be involved in flavonoid metabolism (Kitamura *et al.*, 2004) and signalling (Chen *et al.*, 2007), with many studies also implicating them in a wide range of responses to stress, infection, and plant hormones (Moons, 2005). An underlying problem in studying the functions of these proteins by classical molecular genetic methods is that multiple members of this large gene family can have identical roles (Alfenito *et al.*, 1998), with this redundancy concealing function on gene disruption (Bouché and Bouchez, 2001).

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As an alternative to genetic studies, it has been of interest to use a combination of biochemical and histochemical methods to investigate the functions of individual GSTs from *Arabidopsis*. As a first step, the members of this superfamily of genes encoding functional GSH-dependent transferases and peroxidases needed to be identified. At the next level of enquiry, it was then helpful to know where individual GSTs are expressed in the plant cell, as that could focus the future search for likely physiologically relevant substrates or binding partners. The importance of understanding the sub-cellular localization of GSTs when defining the function of these proteins has recently been demonstrated. Thus, when maize tau class GSTs were expressed in bacteria they were found to interact with porphyrinogen intermediates of haem biosynthesis, catalysing their GSH conjugation (Dixon *et al.*, 2008). Similarly, the transplastomic expression of these proteins in tobacco caused similar changes in porphyrin metabolism. However, despite the apparent selectivity of these GSTs for binding and conjugation of porphyrin intermediates, this does not appear to be their function *in planta* due to their exclusive expression in the cytosol where they are physically separated from mainstream tetrapyrrole biosynthesis in the chloroplast (Tanaka and Tanaka, 2007).

Relatively little attention has been paid to the cellular localization of plant GSTs, as the majority lack obvious sub-cellular targeting sequences and are therefore predicted to accumulate in the cytosol. In *Arabidopsis*, exceptions to this are seen with members of the DHAR (e.g. DHAR3) and lambda (GSTL2) classes where unambiguous N-terminal targeting peptides direct these proteins to the plastid/mitochondrion (Dixon *et al.*, 2002a). The phi class GSTF8 also contains a chloroplast-targeting peptide, though differential mRNA splicing leads to the majority of the derived transcripts lacking this signal, giving cytosolic protein (Thatcher *et al.*, 2007). Despite their predicted cytosolic localization, several proteomic studies in *Arabidopsis* and other plants have consistently shown that various organellar preparations contain high titres of specific GSTs. For example, comprehensive proteomic analyses in *Arabidopsis* have identified GSTs F2, F8, F9, F10, U19, U20, L2, and DHAR3 in the chloroplast (Zybailov *et al.*, 2008), GSTF5 and GSTF6 in the mitochondrion (Heazlewood *et al.*, 2004), and GSTs F2, F6, F7, F9, and F10 in the vacuole (Carter *et al.*, 2004). Earlier studies in other plants have also reported specific GSTs accumulating in the nucleus (Takahashi *et al.*, 1995) and apoplast (Flury *et al.*, 1996). The presence of these GSTs in organelles may in part be due to cross-contamination from the cytosol, where these hydrophobic proteins can be present at high concentrations (Sappl *et al.*, 2004). Alternatively, it is also possible that these GSTs associate with different sub-cellular fractions as a consequence of their association with other proteins or substrates/ligands and as such an understanding of their sub-cellular localization could be very important in unravelling their endogenous function.

Reported here are the cloning and expression of the GST superfamily from *Arabidopsis* and the functional screening

of the recombinant proteins of currently unknown function for GSH-dependent enzyme activities. The subcellular localization of *Arabidopsis* GST family members which lack obvious plastid/mitochondrial targeting sequences has been examined by generating N-terminal green fluorescent protein (GFP) fusions and determining their localization using confocal microscopy after transiently expressing the fusions in *Nicotiana benthamiana*. A total of 18 family members were selected based on (i) a careful examination of the respective sequences of these proteins to look for C-terminal targeting domains and (ii) proteomics literature suggesting that 'cytosolic' *Arabidopsis* GSTs, or orthologues in other plants, were associated with specific organelles. In each case, a library of GSTs cloned into a custom *Strep*-tagged bacterial pET-derived expression plasmid was used, which allowed for the straightforward sub-cloning of sequences into the plant transformation binary vector.

Materials and methods

Vectors

The expression vectors pET-STRP3 and BIN-STRP3 were synthesized as described (Dixon *et al.*, 2008; Supplementary data available at JXB online). For plant expression of N-terminal GFP fusions, the vector BIN-STRP3 was modified such that a GFP tag was introduced between the *Strep*-tag and the cloned protein. This construct was prepared by amplifying GFP from a plasmid containing smGFP (EMBL accession U70495) (Davis and Vierstra, 1998) using the oligonucleotides GCGCGCCGATCGTGAGTAAAGGA-GAAGAAC and GCGCGCCCTGCAGGATCCTTAAT-TAATTTGTATAGTTCATCCATGC. The PCR product was digested using *PvuI* and *SbfI* and ligated into BIN-STRP3 digested with *PacI* and *SbfI* to give BIN-STRP3-GFP. Vectors (for sequences, see Supplementary data available at JXB online) are available upon request.

Cloning of GSTs

Most GST sequences were PCR-amplified from cDNA prepared from a combination of *Arabidopsis* plants and root cultures (Dixon *et al.*, 2002a), using KOD polymerase (Novagen, Nottingham, UK) and the primers detailed in Table S1 in Supplementary data available at JXB online. To amplify GSTF12, mature *Arabidopsis* plants were placed under high light conditions (25 °C, 200 $\mu\text{E m}^{-2}$) for 48 h prior to RNA extraction and cDNA synthesis. Amplified GST sequences were digested with restriction sites engineered into the primers and cloned into similarly digested pET-STRP3. Sequences were sub-cloned from pET-vectors to BIN-vectors typically using *PacI* and *BstXI* restriction sites (Fig. 1A).

GST expression in *Escherichia coli*

The pET-STRP3 constructs were transformed into *E. coli* strain Tuner(DE3) (Novagen) containing the pRARE plasmid from strain Rosetta (Novagen). Cultures were grown in LB broth containing appropriate antibiotics and

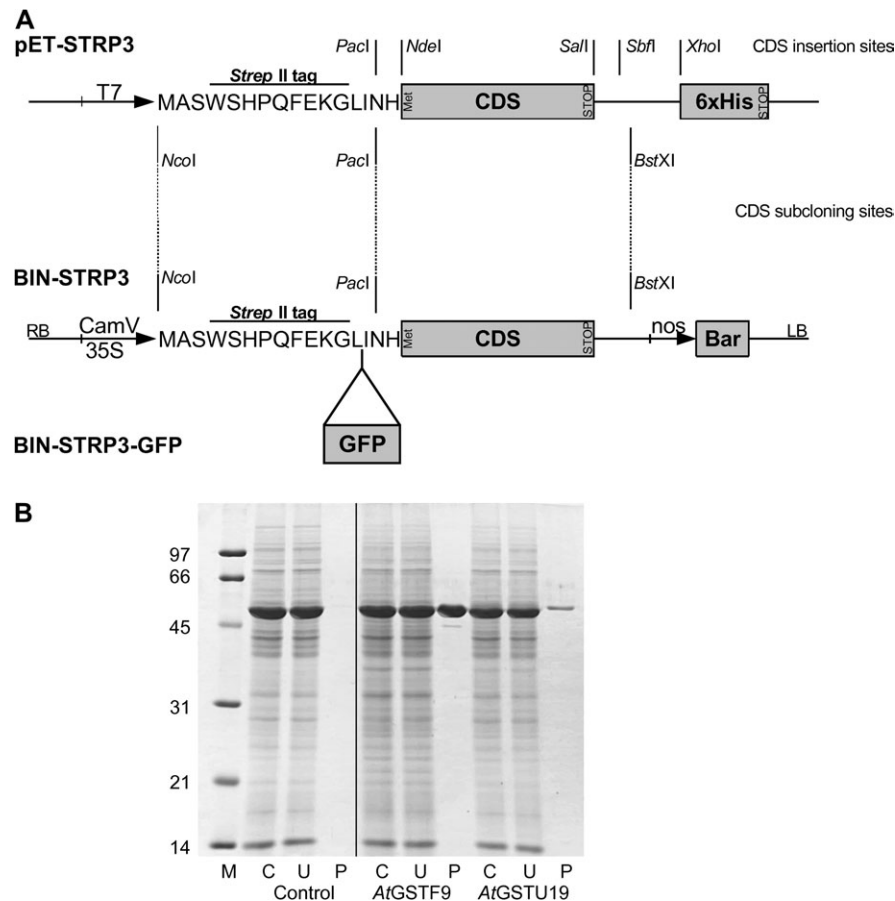


Fig. 1. *Strep*-tag vector design and BIN-STRP3-GFP-mediated expression in plants. (A) Overview of vector design, showing promoters, restriction enzyme sites useful for cloning, and left and right borders (LB, RB) of T-DNA region for the bacterial (pET-STRP3) and plant (BIN-STRP3, BIN-STRP3-GFP) expression vectors. (B) Coomassie Brilliant Blue-stained SDS-PAGE gel showing affinity purification of GFP-GST fusion proteins from *N. benthamiana* leaves infiltrated with BIN-STRP3-GFP constructs. Purifications are shown for the GST fusions as labelled and for a negative control. Lanes for each purification: C, crude protein extract (equivalent to 1 mg FW tissue); U, unbound fraction (equivalent to 1 mg FW tissue); P, purified fraction (equivalent to 100 mg FW tissue). Molecular weight markers (M) are shown with masses (in kDa) as shown. The identities of purified proteins were confirmed by peptide mass fingerprinting. The vertical bar marks the removal of superfluous gel lanes.

0.1 mM IPTG for 16–24 h at 30 °C with shaking at 200 r.p.m. in the dark. Cells were harvested by centrifugation and re-suspended in 5% of the original culture volume in cold (4 °C) HEPES-buffered saline (HBS = 20 mM HEPES-NaOH, 150 mM NaCl, 1 mM EDTA, pH 7.5). After adding DTT (1 mM) and avidin (8 µg ml⁻¹) to remove endogenous biotin and biotinylated proteins, the cells were sonicated and centrifuged, and the lysate loaded (1 ml min⁻¹) onto a 1 ml *Strep*-Tactin macroprep column (Stratagene Scientific Ltd, Soham, UK) pre-equilibrated at 22 °C with HBS. After washing with HBS, recombinant protein was eluted with HBS containing 2.5 mM desthiobiotin and the column regenerated with HBS (10 ml) containing 1 mM 2-(4'-hydroxy-benzeneazo)-benzoic acid. Recombinant protein eluting in a single 1 ml fraction was flash-frozen in 10% v/v glycerol.

Enzyme assays

GST activity was determined with 1-chloro-2,4-dinitrobenzene (CDNB) and benzylisothiocyanate (BITC) as substrates

(Dixon *et al.*, 1998). Glutathione peroxidase (GPOX) activity was determined with cumene hydroperoxide and fatty acid hydroperoxides prepared as described previously (Edwards and Dixon, 2005). Assays with fatty acid hydroperoxides used these substrates at a final concentration of 0.2 mM in the presence of 0.1% (v/v) Triton X-100.

GFP-GST expression in *Nicotiana benthamiana* Domin

GST coding sequences sub-cloned into binary vectors were transformed into *Agrobacterium tumefaciens* GV3101:pMP90 (Koncz and Schell, 1986). Cultures were mixed 1:1 with similarly prepared cultures containing the construct 35S:p19, to provide co-expression of the tomato bushy stunt virus p19 protein which suppressed gene silencing (Voinnet *et al.*, 2003). Cultures were infiltrated into the lower surface of *N. benthamiana* leaves using a syringe (Wroblewski *et al.*, 2005). Transformed leaf tissue was harvested 2–7 d later and used immediately for imaging, or was stored at -80 °C until required for protein

purification. For co-localization studies, similar *A. tumefaciens* cultures containing the constructs px-rk or pm-rk for labelling of peroxisomes and plasma membrane, respectively (Nelson *et al.*, 2007), obtained from NASC (Scholl *et al.*, 2000), were co-infiltrated. Both constructs expressed the red fluorescent protein mCherry, with either a C-terminal SKL motif added for peroxisomal targeting (px-rk) or as a fusion with the plasma membrane aquaporin AtPIP2a (pm-rk) for plasma membrane targeting. After 2–3 d, the transformed leaves were analysed by laser scanning confocal microscopy using a Zeiss LSM 510 Meta instrument with a plan Neofluar $\times 40/\text{NA } 1.3$ oil immersion lens. Where required, tissue samples were counterstained for 5–20 min using 1 mg ml^{-1} propidium iodide and then washed with water before visualization. GFP fusion fluorescence was imaged using excitation with a 488 nm laser, coupled with a 505–530 nm band pass filter, propidium iodide was imaged using a 543 nm laser with a 560-nm-long pass filter, and mCherry fusions were imaged using a 543 nm laser with a 650-nm-long pass filter. For each fluorophore combination, controls lacking each fluorophore in turn confirmed negligible channel crosstalk and autofluorescence (except from chloroplasts).

GFP–GST purification

GSTs were purified from frozen plant tissue after extraction using 4 v/w 100 mM TRIS-Cl pH 7.5 containing 150 mM NaCl, 1 mM EDTA, 10 mM sodium ascorbate, $50 \text{ } \mu\text{g ml}^{-1}$ avidin, $10 \text{ } \mu\text{g ml}^{-1}$ bovine pancreatic DNase I, $10 \text{ } \mu\text{g ml}^{-1}$ bovine pancreatic RNase A, and 5% w/v polyvinylpyrrolidone. After filtration through miracloth (Calbiochem, Nottingham, UK) and clarification by centrifugation (15 000 g, 20 min, 4 °C), the *Strep*-GSTs were affinity purified as described for the bacterially expressed enzymes. Purified proteins were concentrated to $\sim 100 \text{ } \mu\text{l}$ by ultrafiltration through a 10 kDa cutoff membrane (2 ml Vivaspin; Sartorius Stedim UK Ltd, Epsom, UK).

Results

As a first step in studying the whole family of *Arabidopsis* GSTs, the coding sequence for each GST was amplified, with PCR products indicating that the respective gene was indeed transcribed. These clones were then expressed in *E. coli* and purified recombinant proteins assayed to survey for typical GST activities. The cloning and characterization of the GSTZs (Dixon and Edwards, 2006), DHARs, and GSTLs from *Arabidopsis* (Dixon *et al.*, 2002a) has been reported previously. Their further analysis was restricted to their amplification and cloning into the new expression vectors to study their subsequent sub-cellular localization *in planta*. GSTZ1, GSTL1, GSTL2, GSTL3, and DHARs 1–3 were successfully amplified, all without any N-terminal targeting peptides. In the case of DHAR4 and GSTZ2, no amplification products were obtained and, since there is no

evidence of transcripts for these genes, it was concluded that they were probably pseudogenes.

cDNAs encoding all 28 GSTUs and 12 of the GSTFs were amplified from *Arabidopsis*. Of the products obtained, GSTF8 was cloned as the splice variant without a plastidic targeting peptide, since this has been shown to be the major form *in vivo* (Thatcher *et al.*, 2007). GSTF13 could not be amplified and no attempt was made to clone GSTF1 (Bartling *et al.*, 1993), which is not encoded within the sequenced *Arabidopsis* genome (Wagner *et al.*, 2002). The polypeptide sequences of the cloned GSTs matched those predicted from the TAIR 8 genome annotation (www.arabidopsis.org, April 2008 release), with two exceptions. GSTU17 had a frame-shift, but was identical to EMBL cDNA sequence AF288191, while GSTU21 had a 6 bp downstream shift in the start of the second exon. Thus, *gstf13* was the only possible pseudogene of those tested.

For the theta GSTs, three genes lie adjacent to one another on chromosome 5 (*GSTT1*=At5g51210; *GSTT2*=At5g41240; *GSTT3*=At5g41220). *GSTT2* and *GSTT3* are unusual in being annotated as GSTs with a C-terminal Myb-like DNA-binding protein fusion (TAIR release 8). An examination of the C-terminal sequences of the three previously identified *Arabidopsis* GSTTs (Wagner *et al.*, 2002) identified C-terminal SKM or SKI motifs, which would be anticipated to target these proteins to the peroxisome (Reumann, 2004). However, with the *GSTT2* and *GSTT3* genes the C-terminal Myb-like extensions masked the putative peroxisome targeting signal. PCR-based cloning successfully amplified the short forms of all three genes, with *GSTT3* amplified by mis-priming with *GSTT2* primers, which introduced an additional methionine at the N-terminus but did not alter the C-terminus. The resulting amplification products encoded the 28 kDa proteins *GSTT1*, *GSTT2*, and *GSTT3*, respectively (for sequences, see Supplementary data available at *JXB* online). A long splice variant of *GSTT3* could also be amplified, encoding the 68 kDa GST–Myb fusion *GSTT3L*. Although not isolated, splice variants of *GSTT2* encoding the GST–Myb fusion *GSTT2L* have also been reported in the DNA databases.

In total, 51 *Arabidopsis* GSTs were found to be transcribed, with the respective coding sequences recovered by PCR amplification. A custom vector system was developed to provide inducible high level expression of GSTs in recombinant *E. coli*, using an N-terminal *Strep*-tag II (Skerra and Schmidt, 1999). This pET-STRP3 vector was engineered to minimize restriction enzyme incompatibility by utilizing flanking 6 bp cutters for routine use, with additional flanking 8 bp rare cutters introduced for problem sequences (Fig. 1A). Using this vector, all cloned GSTs were expressed in *E. coli*. Focusing on the phi, tau, and theta class enzymes, a total of 41 GSTs could be purified as soluble proteins using *Strep*-Tactin affinity chromatography (Figs S1 and S2 in Supplementary data available at *JXB* online). Only GSTF11 and GSTF12 were undetectable in the soluble fraction. In all cases, MS analysis of purified GSTs showed they had undergone cleavage of the N-terminal methionine, with the revealed alanine residue of the *Strep*-tag oligopeptide

(ASWSHPQFEK) undergoing partial *N*-acetylation (Table S2), an unusual post-translational modification in *E. coli* (Charbaut *et al.*, 2002). Several GSTs were found to co-purify protein contaminants (Figs S1, S2A in Supplementary data available at *JXB* online), which were identified by peptide mass fingerprinting. Notably, GSTU12, GSTT1, and GSTT2 were found to co-purify ribosomal proteins, and GSTF5 co-purified the chaperone GroEL. A number of preparations contained a 70 kDa polypeptide identified as the E1 subunit of pyruvate dehydrogenase, which was therefore presumed to be a non-specific contaminant. For the theta class GSTs, ethidium bromide counterstaining of SDS-PAGE gels showed that GSTT1, GSTT2, and GSTT3L contained nucleic acids, while GSTT3 showed only minor contamination (Fig. S2B).

To test for functionality, recombinant phi, tau, and theta class GSTs were assayed for activity toward typical GST substrates. GSH-conjugating activity was assessed using the model xenobiotic substrate CDNB as well as BITC, the latter being a potential natural GST substrate in cruciferous plants derived from the degradation of glucosinolates (Gil and MacLeod, 1980). GSH-dependent peroxidase (GPOX) activity was routinely determined with cumene hydroperoxide (Edwards and Dixon, 2005). To examine the linkage between enzyme activity and relatedness, the results of these assays are represented with the GSTs clustered on the basis of their polypeptide sequence similarities (Fig. 2). Several of the 41 GSTFs and GSTUs could not be assayed. GSTF11, GSTF12, and GSTF13 could not be cloned, or only expressed as insoluble proteins, while GSTF4, GSTF10, and GSTU15 were only obtained in very low yields. Of the remaining 35 recombinant proteins, 32 showed GSH-dependent enzyme activity, with only GSTF5, GSTF14, and GSTU11 being inactive toward the three substrates tested. Closely related GSTs showed a similar spectrum of enzyme activities, although absolute values for specific activities between enzymes varied. The conjugation of CDNB was the most commonly observed enzyme activity, though it varied over three orders of magnitude within the superfamily (Fig. 2). BITC was a more discriminating GST substrate, being acted on by most tau class enzymes but rarely by the GSTFs. The majority of the GSTs also had some GPOX activity, with GSTU25 having particularly high activity. When the three GSTTs were assayed, these enzymes were shown to be highly active as GPOXs. To explore these activities in greater detail, two fatty acid hydroperoxides were prepared and found to be excellent GPOX substrates. This was in contrast to GSTU25 which, although showing high GPOX activity towards cumene hydroperoxide, showed little activity toward the fatty acid hydroperoxides that are likely substrates of GPOXs *in planta* (Bartling *et al.*, 1993).

Generation and testing of custom plant expression vectors

Novel binary vectors were constructed to complement the bacterial expression vector, allowing *Agrobacterium*-mediated

constitutive expression of *Strep*-tagged proteins in plants (Fig. 1A). BIN-STRP3 allowed plant-based expression of the same tagged protein as given by pET-STRP3 in bacteria, and BIN-STRP3-GFP provided an additional N-terminal GFP tag for visualization studies while retaining the *Strep*-tag for easy gel-based detection and/or purification if required. For example, GSTs could be recovered after transient expression in *N. benthamiana* and shown to be intact (Fig. 1B) and fluorescent, giving confidence that *in vivo* fluorescence data was not compromised by proteolytic cleavage of the GFP-GST fusion. Both vectors facilitated sub-cloning from the corresponding pET-STRP3 expression vector by incorporating compatible rare-cutting restriction sites.

GSTTs as Arabidopsis GST family members with C-terminal targeting motifs

Having established a tractable expression system which allowed for both the functional expression of GSTs in *E. coli* and the definition of their sub-cellular localization *in planta* it was then of interest to determine whether the information derived from these two experimental systems could provide insight into the roles of GST family members. Examining the functional activities of GSTs and the potential for differential sub-cellular localization, it was apparent that the GSTTs represented a class of proteins which had conserved enzyme activities and the potential for targeting to different subcellular organelles. Thus, GSTs T1, T2, and T3 contained C-terminal domains corresponding to likely peroxisomal targeting motifs (C-terminal SKI, SKM, and SKM motifs, respectively), whereas GSTT2L and GSTT3L contained polypeptide extensions resembling Myb transcription factors which were more likely to direct the fusion proteins to the nucleus. To investigate their sub-cellular localization, GFP fusions of GSTT1, GSTT2, GSTT3 (short form), and GSTT3L (transcription factor fusion) were transiently expressed in *Nicotiana benthamiana*, and their localization then monitored by laser scanning confocal microscopy. GFP-GSTT1 (Fig. 3A), GFP-GSTT2 (Fig. 3B), and GFP-GSTT3 (Fig. 3C) were each confined to small organelles which, on the basis of their mobility, number, and size, together with the putative C-terminal targeting sequence of these GSTs, were assumed to be peroxisomes. This was confirmed (Fig. S3 in Supplementary data available at *JXB* online) by co-localization with the peroxisomal marker px-rk (Nelson *et al.*, 2007). By contrast, GFP-GSTT3L localized solely to the nucleus (Fig. 3D, Fig. S4), giving a punctate expression pattern and showing exclusion from the nucleolus.

Localization of other plant GSTs

In addition to the theta class enzymes, a range of phi (F2, F6, F8, F9, and F12) and tau (U2, U7, U9, U11, U12, U19, and U28) GSTs were also tested for sub-cellular localization using GFP tagging. In each case, these GSTs lacked characterized C-terminal targeting motifs, and the N-terminal transit peptide of GSTF8 was removed prior to

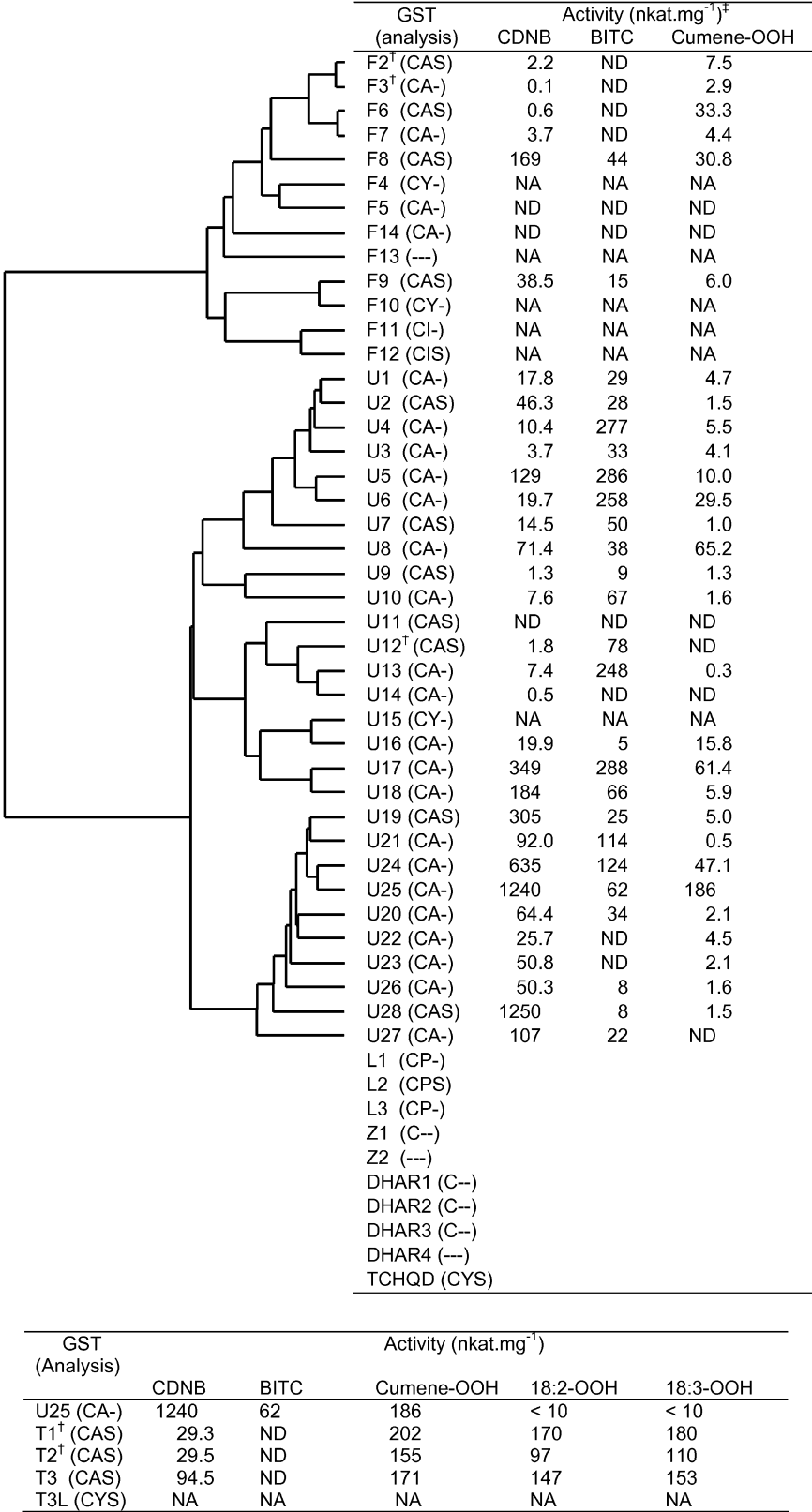


Fig. 2. Phylogenetic tree and enzyme activities of bacterially expressed recombinant *Arabidopsis* phi (F) and tau (U) class GSTs assayed with 1-chloro-2,4-dinitrobenzene (CDNB), benzyl isothiocyanate (BITC), and cumene hydroperoxide (cumene-OOH). Also shown are theta (T) class GSTs (with GSTU25 for comparison), assayed for GPOX activity with the additional substrates (13S,9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid (18:2-OOH) and (13S,9Z,11E,15Z)-13-hydroperoxy-9,11,15-octadecatrienoic acid (18:3-OOH). Each GST name is suffixed with a key describing the associated analysis: C, successfully cloned; A, purified recombinant protein assayed; P, recombinant protein purified but not assayed; I, recombinant protein totally insoluble; Y, recombinant protein not available in

testing. GFP–GST fusions of all the GSTFs and GSTs U2, U7, U9, U11, U19, and U28 all localized to the cytosol. In most cases GFP fusions were also found at low levels in the nucleus, presumably due to passive diffusion, as illustrated by GFP–GSTF2 and GFP–GSTU19 (Fig. 4A, B), with both of these GSTs being described as abundantly expressed proteins in proteomic studies (Sappl *et al.*, 2004; Smith *et al.*, 2004). GFP fusions of GSTs U7, U9, U17, and U28 were practically absent from the nuclei (Fig. 4C). By contrast, GFP–GSTU12 localized entirely to the nucleus (Fig. 4D), confirmed by counterstaining with propidium iodide. As compared with related GSTs, examination of the GSTU12 polypeptide sequence revealed an N-terminal extension of 25 amino acid residues containing a putative nuclear localization signal (KKRKK), although it is beyond the scope of this work to test whether this signal is necessary and sufficient for nuclear import. Sub-cellular localization studies were then extended to other GSTs containing unusual sequence motifs. The lambda protein

GSTL2 (without its N-terminal signal peptide) was selected since it has a potential peroxisomal targeting sequence (ARL) at its C-terminus. GFP–GSTL2 was found to localize to both the cytosol and peroxisomes (Fig. 4E), confirming the presence of a peroxisomal targeting signal. The unusual GST superfamily member TCHQD was also studied. GFP–TCHQD localized to the plasma membrane (Fig. 4F), co-localizing (Fig. S3) with the diagnostic marker pm-rk (Nelson *et al.*, 2007). Table 1 summarizes the distribution of non-cytosolic *Arabidopsis* GSTs.

Discussion

Differential targeting of the Arabidopsis GST superfamily

The *Arabidopsis* genome contains 54 identified members of the GST superfamily. Of these 51 are transcribed and at least 41 encode functional GSH-dependent enzymes when

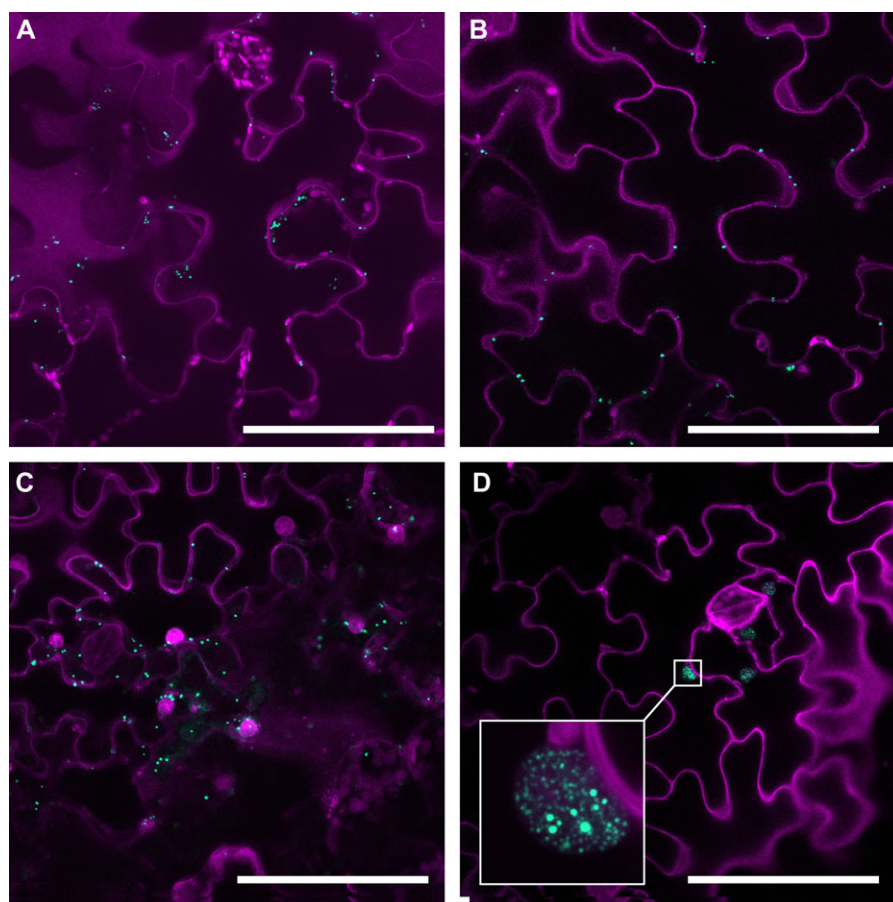


Fig. 3. Laser scanning confocal micrographs showing intracellular localization of N-terminally GFP-tagged GSTs transiently expressed in *N. benthamiana* (green channel), with counterstaining with propidium iodide (magenta channel). GSTs shown are GSTT1 (A), GSTT2 (B), GSTT3 (C), and GSTT3L (D). Scale bars represent 100 μ M.

sufficient yield/purity for assay; S, subcellular localization studied; a dash=analysis not performed. ND, No activity detected; NA, not assayed. [†] GST concentration overestimated due to co-purifying chromophores, so activity is underestimated. [‡] Variation in the replicates from the mean values quoted was <10% in all cases.

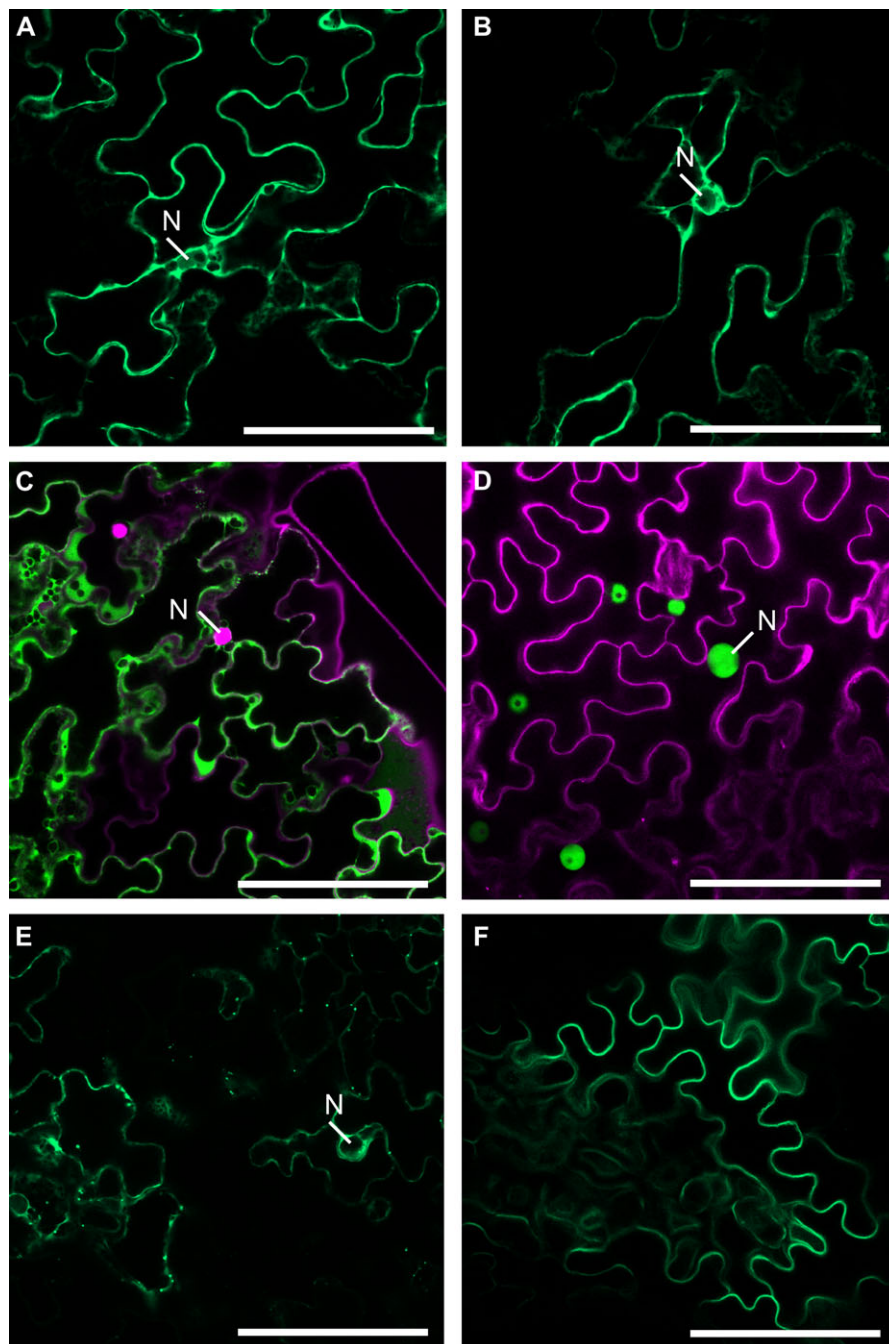


Fig. 4. Laser scanning confocal micrographs showing intracellular localization of N-terminally GFP-tagged GSTs transiently expressed in *N. benthamiana* (green channel), with some panels counterstained with propidium iodide (magenta channel). GSTs shown are GSTF2 (A), GSTU19 (B), GSTU28 (C; nuclei and cell walls counterstained), GSTU12 (D; cell walls counterstained); GSTL2 (E), and TCHQD (F). N, nucleus. Scale bars represent 100 μ M.

assayed as GSTs or GPOXs (GSTFs, GSTUs, GSTTs; Fig. 2) thioltransferases or reductases (DHARs and GSTLs) (Dixon *et al.*, 2002a) or isomerases (GSTZs) (Dixon and Edwards, 2006). Among the GSTFs, GSTUs, and GSTTs it was clear from the enzyme screen (Fig. 2) that family members of related sequence have a broadly similar spectrum of activities as GSTs and GPOXs when assayed with model substrates. While it is conceivable that each enzyme has highly selective activities directed toward a well-defined group of natural substrates, the present results

would suggest that it is more likely that there is a substantial overlap of activities and functional redundancy within the superfamily. Expression of particular GST isoenzymes in different plant organs and tissues and in response to different stimuli (e.g. constitutive Vs stress-inducible) have been proposed as reasons to explain this apparent redundancy (Edwards *et al.*, 2000; Frova, 2003). The results presented here suggest that there is an additional reason for apparent functional redundancy within the family; namely, multiple GSTs with similar activities may be required to

Table 1. Summary of GSTs found to accumulate outside the cytosol, with proposed targeting signal
Data are based on N-terminal GFP fusions, except where indicated.

GST (gene)	Location	Likely signal
GSTT1 (At5g41210)	Peroxisome	C-term SKI
GSTT2 (At5g41240)	Peroxisome	C-term SKM
GSTT3 (At5g41220)	Peroxisome	C-term SKM
GSTT3L (At5g41220)	Nucleus (speckles?)	C-term Myb-like domain
GSTU12 (At5g41210)	Nucleus	Nuclear localization signal (KKRKK) in N-term extension
GSTL2 (At3g55040)	Chloroplast*	N-term targeting peptide*
	Peroxisome	C-term ARL
DHAR3 (At5g16710)	Chloroplast*	N-term targeting peptide*
TCHQD (At1g77290)	Plasma membrane	Unknown

* Previously identified targeting peptides, giving an assumed chloroplastic location (Dixon *et al.*, 2002a).

perform similar functions in different subcellular compartments. In this respect, the GSTTs serve as an excellent example.

The three GSTTs in *Arabidopsis* have clearly arisen through gene duplication (Cannon *et al.*, 2004), with the enzymes showing conservation in both sequence and enzyme activity. Thus, all three GSTTs were highly active as GPOXs when assayed with organic hydroperoxides. The GFP–fusion protein expression studies *in planta* showed that, whereas GSTT1 and the short splice variants of GSTT2 and GSTT3 would express their GPOX activity in the peroxisomes, GSTT3L, with its C-terminal Myb-like extension, was directed to the nucleus where it accumulated in distinct nuclear substructures to give a punctate appearance. This distribution closely resembles that of nuclear speckles, thought to be storage sites for pre-mRNA splicing-related proteins and also other proteins (Shaw and Brown, 2004). Although theta class GSTs have been described in mammals (Jowsey *et al.*, 2001), insects (Ding *et al.*, 2003), and fungi (Bryant *et al.*, 2006), these enzymes have not attracted the same level of attention as other GST classes. Mammalian GSTTs characteristically exhibit dehalogenase activity, are highly active GPOXs, and show low affinity binding of GSH and little transferase activity toward CDNB (Landi, 2000). Similarly, plant GSTTs are also highly active as GPOXs, while showing limited GST activities (Dixon *et al.*, 1999). However, whereas GSTTs in animals are stress responsive proteins (Landi, 2000), the respective genes in *Arabidopsis* were unresponsive to chemical and abiotic stress treatments (Dixon *et al.*, 2002a; Wagner *et al.*, 2002). In *Arabidopsis*, the GSTTs were expressly targeted to either the peroxisomes or the nucleus. The expression of GPOX-active GSTTs in peroxisomes presumably reflects the oxidative environment within this compartment which leads to the generation of phytotoxic fatty acid hydroperoxides, which are substrates of these enzymes (Fig. 2). Thus, the peroxisomal localization of GSTT1, GSTT2, and GSTT3 would most likely be associated with a protective function in maintaining the integrity

of the peroxisomal membranes. Theta class GSTs in other plants also have C-terminal motifs that are likely to result in peroxisomal targeting, including GSTT proteins from rice (GenBank AAK98534), soybean (GenBank AAG34813), and *Medicago truncatula* (GenBank ABE92134). However, GSTTs in animals and fungi have no such obvious targeting sequences and have not been reported to be associated with peroxisomes. This is in contrast to other classes of GSTs, such as the kappa class proteins in mammals (Morel *et al.*, 2004), and omega-class GSTs in *Saccharomyces cerevisiae* (Barreto *et al.*, 2006), which are known to be targeted to the peroxisomes. This suggests that, despite their sequence and apparent functional conservation across the phyla, GSTTs in plants have been recruited to perform unique roles in peroxisomal metabolism.

The accumulation of GSTTs in the nucleus is less easy to rationalize. The localization of GSTT3L to this organelle is clearly determined by the presence of the unusual C-terminal extension which resembles a Myb DNA-binding protein. GSTTs have previously been reported in the nucleus in animal cells. Thus, in hepatocytes, GSTTs are expressed evenly in both the cytosol and the nucleus (Liteplo *et al.*, 1998; Sherratt *et al.*, 1998), with one report of preferential nuclear expression in specialized murine tissues (Quondamatteo *et al.*, 1998). This suggests that GSTTs may have originally evolved functions which are required both in the cytosol and in the nucleus. In plants, the latent ability of GSTTs to bind to nucleic acids may offer a clue as to the functional significance of the selective localization of GSTT isoforms. When affinity purified from *E. coli*, both GSTT1 and GSTT2 preparations were heavily contaminated with nucleic acid and ribosomal polypeptide components (Fig. S2 in Supplementary data available at JXB online). It is likely that the RNA-binding properties of these proteins are due to their basic nature (calculated pI of pH 9.50 and pH 9.32 for GSTT1 and GSTT2, respectively) giving rise to non-specific interactions with nucleic acids. The short splice variant of GSTT3 did not bind nucleic acids when expressed in bacteria, presumably due to its less basic nature (pI 8.89), whereas GSTT3L with its C-terminal extension showed obvious nucleic acid contamination. These results clearly demonstrate that GSTT3L binds nucleic acids as a consequence of its transcription factor-like domain (although this is unlikely to be as clear-cut for GSTT2L since GSTT2 retains RNA binding). This may suggest that for GSTT3L to exhibit a selective function in the nucleus there has been selective pressure to decrease the non-specific binding of nucleic acid to the GSTT domain, as achieved through the replacement of basic residues. Instead, the ability to selectively bind to nucleic acids has been introduced through the fusion with the Myb-like domain. While the function of GSTT3L is yet to be determined, it is speculated that these proteins either play a redox-sensitive role in controlling transcription, or are involved in specialized nucleic acid repair. With respect to the latter hypothesis, it is known that mammalian GSTTs reduce DNA hydroperoxides formed *in vitro* through X-ray radiation damage (Tan *et al.*, 1988). It is therefore possible that

targeted by the C-terminal Myb-like domain, the function of GSTT3L is to repair actively transcribed DNA segments which are subject to elevated levels of oxidative damage. Examination of other plant GSTT sequences failed to identify similar fusions in other species, so the *Arabidopsis* GSTT fusions may have a very specialized role that most plants either do not require or have an alternative system in place.

Localization of other GSTs

Most of the GSTs tested accumulated in the cytosol, as expected based on their lack of any obvious targeting sequences. This does suggest that members of this cytosolic group of GSTs which are reported to associate with mitochondrial, chloroplastic, and vacuolar proteomes do so as a result of contamination of these organelles with these relatively abundant and hydrophobic proteins (Carter *et al.*, 2004; Heazlewood *et al.*, 2004; Zybailov *et al.*, 2008). Similar conclusions have been made regarding GSTA1-1 in rats, which though known to be expressed in the cytosol accumulate to high levels in the outer nuclear envelope (Stella *et al.*, 2007). While this may explain the presence of relatively abundant cytosolic GSTs as proteome components of organelles in *Arabidopsis*, it is also possible that family members which are present in lower abundance in the cell are carried into organelles through interactions with explicitly targeted proteins. Intriguingly, some, but not all of the predominantly cytosolic GFP–GST fusions were also present at low levels in the nucleus, consistent with their import by passive diffusion. It is possible that the GFP–GSTUs which solely localized to the cytosol did so as a consequence of the size of the fusion protein precluding nuclear import, with the smaller native GSTs more likely to enter the pores. Certainly the GFP–GST fusions appeared to be processed as fully functional polypeptides which, in view of their stability and yield, were most likely expressed as homodimers (Fig. 1). In any event, the ability of several of the GSTs to move between the nucleus and cytosol should be taken into account when considering their potential ligands and roles in inter-compartmental transport and signalling.

The present studies have shown that several GSTs previously assumed to be cytosolic are targeted elsewhere. GSTU12 was clearly nuclear-localized and, to date, the only other known tau class GST known to accumulate in this compartment is ParA from tobacco (Takahashi *et al.*, 1995). Unlike GSTU12, ParA has no obvious localization signal and the functional significance of this nuclear targeting is unknown. The roles for GSTU12 being directed to the nucleus must be distinct from that inferred for GSTT3L. Unlike GSTT3, GSTU12 has no activity as a GPOX and therefore would be unlikely to be involved in reducing either DNA- or lipid-derived hydroperoxides. This GST may instead function to regulate the activity of transcriptional regulators within the nucleus, with a precedent being the interaction of human GSTP1-1 with c-Jun N-terminal kinase (Wang *et al.*, 2001).

The other intriguing subcellular localization of the *Arabidopsis* GST family members was seen in the selective accumulation of TCHQD at the plasma membrane. Previously, only GSTF2 has been reported to associate with the plasma membrane in *Arabidopsis* (Smith *et al.*, 2003), though in the present study this localization was not observed (Fig. 4A). This difference has not been investigated further but could be due to the lack of appropriate hydrophobic GSTF2 ligands in tobacco leaves that promote membrane association in *Arabidopsis* roots. The functional significance of membrane-localized TCHQD is unclear, not least because the respective recombinant enzyme had no activity as a GST or GPOX. This GST family member showed similarity (25% identity) to the TCHQD identified in *Sphingobium chlorophenolicum*, an enzyme which catalyses the reductive dehalogenation of TCHQ and trichlorohydroquinone, which are key steps in the degradation of the pesticide pentachlorophenol (Warner *et al.*, 2008). The endogenous functions of this enzyme in bacteria are unknown, but its evolutionary origins and enzymic mechanism are related to the GSTZ isomerases (Anandarajah *et al.*, 2000). In bacteria, TCHQDs are soluble proteins, and it is most likely that the membrane-associated orthologue in *Arabidopsis* has been recruited for quite different functions. Significantly, the knowledge of the subcellular localization of this GST and the development of vectors allowing for the expression of *Strep*-tagged fusion proteins in *planta* now provide a set of unique tools to probe for the binding partners and ligands of this protein and other family members.

Supplementary data

sequences.txt FASTA-format nucleotide and polypeptide sequences of GST coding sequences cloned into pET-STRP3.

vectors.txt FASTA-format nucleotide sequences of vectors pET-STRP3, BIN-STRP3, and BIN-STRP3–GFP.

Table S1. Primers used for PCR amplification of GSTs.

Table S2. Theoretical and measured masses for purified *Strep*-tagged GSTs expressed in *E. coli* and plants.

Figure S1. Coomassie Brilliant Blue-stained SDS–PAGE gels of *Strep*-tagged *Arabidopsis* phi and tau class GSTs purified from over-expressing *E. coli*.

Figure S2. Coomassie Brilliant Blue-stained SDS–PAGE gels of *Strep*-tagged *Arabidopsis* theta class GSTs purified from over-expressing *E. coli*, showing RNA binding.

Figure S3. Laser scanning confocal micrographs showing colocalization of GFP–GST fusions with organellar markers.

Figure S4. Laser scanning confocal micrographs showing localization of GFP–GSTT3L to the nucleus, counter-stained with propidium iodide.

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References

- Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V.** 1998. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. *The Plant Cell* **10**, 1135–1149.
- Anandarajah K, Kiefer PM, Donohoe BS, Copley SD.** 2000. Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol. *Biochemistry* **39**, 5303–5311.
- Barreto L, Garcerá A, Jansson K, Sunnerhagen P, Herrero E.** 2006. A peroxisomal glutathione transferase of *Saccharomyces cerevisiae* is functionally related to sulfur amino acid metabolism. *Eukaryotic Cell* **5**, 1748–1759.
- Bartling D, Radzio R, Steiner U, Weiler EW.** 1993. A glutathione S-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*. *European Journal of Biochemistry* **216**, 579–586.
- Bouché N, Bouchez D.** 2001. Arabidopsis gene knockout: phenotypes wanted. *Current Opinion in Plant Biology* **4**, 111–117.
- Bryant D, Cummins I, Dixon DP, Edwards R.** 2006. Cloning and characterization of a theta class glutathione transferase from the potato pathogen *Phytophthora infestans*. *Phytochemistry* **67**, 1427–1434.
- Cannon SB, Mitra A, Andrew B, et al.** 2004. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biology* **4**, 1–21.
- Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV.** 2004. The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. *The Plant Cell* **16**, 3285–3303.
- Charbaut E, Redeker V, Rossier J, Sobel A.** 2002. N-terminal acetylation of ectopic recombinant proteins in *Escherichia coli*. *FEBS Letters* **529**, 341–345.
- Chen I-C, Huang I-C, Liu M-J, Wang Z-G, Chung S-S, Hsieh H-L.** 2007. Glutathione S-transferase interacting with far-red insensitive 219 is involved in phytochrome A-mediated signaling in Arabidopsis. *Plant Physiology* **143**, 1189–1202.
- Davis SJ, Vierstra RD.** 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Molecular Biology* **36**, 521–528.
- Ding YC, Ortellì F, Rossiter LC, Hemingway J, Ranson H.** 2003. The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* **4**, 1–16.
- Dixon DP, Cole DJ, Edwards R.** 1998. Purification, regulation and cloning of a glutathione transferase (GST) from maize resembling the auxin-inducible type-III GSTs. *Plant Molecular Biology* **36**, 75–87.
- Dixon DP, Cole DJ, Edwards R.** 1999. Identification and cloning of AtGST 10 (Accession Nos. AJ131580 and AJ132398), members of a novel type of plant glutathione transferases. *Plant Physiology* **119**, 1568–1568.
- Dixon DP, Davis BG, Edwards R.** 2002a. Functional divergence in the glutathione transferase superfamily in plants: identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **277**, 30859–30869.
- Dixon DP, Edwards R.** 2006. Enzymes of tyrosine catabolism in *Arabidopsis thaliana*. *Plant Science* **171**, 360–366.
- Dixon DP, Laphorn A, Edwards R.** 2002b. Plant glutathione transferases. *Genome Biology* **3**, reviews3004.1–reviews3004.10.
- Dixon DP, Laphorn A, Madesis P, Mudd EA, Day A, Edwards R.** 2008. Binding and glutathione conjugation of porphyrinogens by plant glutathione transferases. *Journal of Biological Chemistry* **283**, 20268–20276.
- Edwards R, Dixon DP.** 2005. Plant glutathione transferases. *Methods in Enzymology* **401**, 169–186.
- Edwards R, Dixon DP, Walbot V.** 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends in Plant Science* **5**, 193–198.
- Flury T, Wagner E, Kreuz K.** 1996. An inducible glutathione S-transferase in soybean hypocotyl is localized in the apoplast. *Plant Physiology* **112**, 1185–1190.
- Frova C.** 2003. The plant glutathione transferase gene family: genomic structure, functions, expression and evolution. *Physiologia Plantarum* **119**, 469–479.
- Gil V, MacLeod AJ.** 1980. Benzylglucosinolate degradation in *Lepidium sativum*: effects of plant age and time of autolysis. *Phytochemistry* **19**, 1365–1368.
- Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH.** 2004. Experimental analysis of the arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *The Plant Cell* **16**, 241–256.
- Jowsey IR, Thomson AM, Flanagan JU, Mudock PR, Moore GBT, Meyer DJ, Murphy GJ, Smith SA, Hayes JD.** 2001. Mammalian class Sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D₂ synthases. *Biochemical Journal* **359**, 507–516.
- Kitamura S, Shikazono N, Tanaka A.** 2004. TRANSPARENT TESTA 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in Arabidopsis. *The Plant Journal* **37**, 104–114.
- Koncz C, Schell J.** 1986. The promoter of T L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Molecular Genetics and Genomics* **204**, 383–396.
- Landi S.** 2000. Mammalian class theta GST and differential susceptibility to carcinogens: a review. *Mutation Research* **463**, 247–283.
- Liteplo RG, Long GW, Meek ME.** 1998. Relevance of carcinogenicity bioassays in mice in assessing potential health risks associated with exposure to methylene chloride. *Human & Experimental Toxicology* **17**, 84.
- Moons A.** 2005. Regulatory and functional interactions of plant growth regulators and plant glutathione S-transferases (GSTs). *Plant Hormones* **72**, 155–202.
- Morel F, Rauch C, Petit E, Piton A, Theret N, Coles B, Guillouzo A.** 2004. Gene and protein characterization of the human

glutathione S-transferase kappa and evidence for a peroxisomal localization. *Journal of Biological Chemistry* **279**, 16246–16253.

Nelson BK, Cai X, Nebenführ A. 2007. A multicolored set of *in vivo* organelle markers for co-localization studies in Arabidopsis and other plants. *The Plant Journal* **51**, 1126–1136.

Quondamatteo F, Schulz TG, Bunzel N, Hallier E, Herken R. 1998. Immunohistochemical localization of glutathione S-transferase-T1 in murine kidney, liver, and lung. *Histochemistry and Cell Biology* **110**, 417–423.

Reumann S. 2004. Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses. *Plant Physiology* **135**, 783–800.

Sappl PG, Oñate-Sánchez L, Singh KB, Millar AH. 2004. Proteomic analysis of glutathione S-transferases of *Arabidopsis thaliana* reveals differential salicylic acid-induced expression of the plant-specific phi and tau classes. *Plant Molecular Biology* **54**, 205–219.

Scholl RL, May ST, Ware DH. 2000. Seed and molecular resources for Arabidopsis. *Plant Physiology* **124**, 1477–1480.

Shaw PJ, Brown JWS. 2004. Plant nuclear bodies. *Current Opinion in Plant Biology* **7**, 614–620.

Sherratt PJ, Manson MM, Thomson AM, Hissink EAM, Green T, Hayes JD. 1998. Increased bioactivation of dihaloalkanes in rat liver due to induction of class theta glutathione S-transferase T1-1. *Biochemical Journal* **335**, 619–630.

Skerra A, Schmidt TGM. 1999. Applications of a peptide ligand for streptavidin: the Strep-tag. *Biomolecular Engineering* **16**, 79–86.

Smith AP, DeRidder BP, Guo WJ, Seeley EH, Regnier FE, Goldsbrough PB. 2004. Proteomic analysis of *Arabidopsis* glutathione S-transferases from benoxacor- and copper-treated seedlings. *Journal of Biological Chemistry* **279**, 26098–26104.

Smith AP, Nourizadeh SD, Peer WA, Xu J, Bandyopadhyay A, Murphy AS, Goldsbrough PB. 2003. *Arabidopsis* AtGSTF2 is regulated by ethylene and auxin, and encodes a glutathione S-transferase that interacts with flavonoids. *The Plant Journal* **36**, 433–442.

Stella L, Pallottini V, Moreno S, et al. 2007. Electrostatic association of glutathione transferase to the nuclear membrane:

evidence of an enzyme defense barrier at the nuclear envelope. *Journal of Biological Chemistry* **282**, 6372–6379.

Takahashi Y, Hasezawa S, Kusaba M, Nagata T. 1995. Expression of the auxin-regulated *parA* gene in transgenic tobacco and nuclear localization of its gene product. *Planta* **196**, 111–117.

Tan KH, Meyer DJ, Gillies N, Ketterer B. 1988. Detoxification of DNA hydroperoxide by glutathione transferases and the purification and characterization of glutathione transferases of the rat liver nucleus. *Biochemical Journal* **254**, 841–845.

Tanaka R, Tanaka A. 2007. Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology* **58**, 321–346.

Thatcher LF, Carrie C, Andersson CR, Sivasithamparam K, Whelan J, Singh KB. 2007. Differential gene expression and sub-cellular targeting of Arabidopsis glutathione S-transferase F8 is achieved through alternative transcription start sites. *Journal of Biological Chemistry* **282**, 28915–28928.

Voinnet O, Rivas S, Mestre P, Baulcombe D. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949–956.

Wagner U, Edwards R, Dixon DP, Mauch F. 2002. Probing the diversity of the *Arabidopsis* glutathione S-transferase gene family. *Plant Molecular Biology* **49**, 515–532.

Wang T, Arifoglu P, Ronai Z, Tew KD. 2001. Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *Journal of Biological Chemistry* **276**, 20999–21003.

Warner JR, Behlen LS, Copley SD. 2008. A trade-off between catalytic power and substrate inhibition in TCHQ dehalogenase. *Biochemistry* **47**, 3258–3265.

Wroblewski T, Tomczak A, Michelmore R. 2005. Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnology Journal* **3**, 259–273.

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ. 2008. Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE* **3**, e1994 1–19.